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Construction of Broad-Host-Range Plasmid Vectors for Easy Visible Selection and Analysis of Promoters

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We have constructed a series of broad-host-range plasmids which use "visual screens" to detect promoter activity. These plasmids contain the pMB1 and pRO1600 origins of replication and are capable of replicating in a wide range of gram-negative bacteria. The genes encoding β-galactosidase and alkaline phosphatase from *Escherichia coli* and bacterial luciferase from *Vibrio harveyi* supply the promoterless indicator genes. The constructs were tested in *E. coli* and *Pseudomonas aeruginosa*.

The lack of versatile small plasmid vectors for use in *Pseudomonas* spp. led us to construct a set of plasmids for general cloning and promoter selection in *Pseudomonas* spp. and *Escherichia coli* (9). These were based on the pRO1600 replicon, which can be maintained in a wide variety of gram-negative bacteria (18). Promoter selection was dependent on cloning DNA sequences upstream of promoterless tetracycline (*tetA*) (6) and chloramphenicol (*cat*) (23) antibiotic resistance genes. These vectors have been used to clone promoters from *Pseudomonas aeruginosa* bacteriophages φPLS27 (1, 9) and D3 (10, 14; M. A. Farinha, B. J. Allan, S. Ronald, and A. M. Kropinski, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, H-114, p. 188) and also to dissect the promoters of the *regA* gene, which controls the expression of exotoxin A in *P. aeruginosa* (D. Storey, personal communication). The use of antibiotic resistance for promoter selection has proven difficult in several respects. The selection of weak or temporally regulated promoters is tricky, since the cells often do not survive the antibiotic challenge. Furthermore, the need to use high concentrations of antibiotics when working with *Pseudomonas* spp. also limits the utility of these constructs. Finally, the expense and limited sensitivity of available assay systems for quantitating promoter activity are major drawbacks in using these vectors.

This article introduces a new series of promoter selection plasmids which have been developed to overcome the problems of the first-generation constructs. They utilize promoterless *lacZ* (β-galactosidase) and *phoA* (alkaline phosphatase) genes from *E. coli* and the *luxAB* (luciferase) genes from *Vibrio harveyi*. The activities of the products of these genes can be visually assayed with a high degree of sensitivity and accuracy (3, 13, 16).

Construction of plasmids. pQF40 is a tetracycline promoter-probe vector derived from pQF10, a deletion derivative of pRO1614 (9, 18). This plasmid was used as the basic replicon for constructing pQF50 (*lacZ*), pQF60 (*phoA*), and pQF70 (*luxAB*).

pQF50 utilizes the promoterless *lacZ* gene derived from pCB267 (21). In the latter construct, the first 15 bases of the *lacZ* gene have been replaced with the Shine-Dalgarno sequence and the first 12 bases of the *E. coli* lipoprotein (*lpp*) gene (12, 17, 20). This region also contains translational stop codons in all three reading frames. The hybrid β-galactosidase is enzymatically indistinguishable from the native protein, which is 1 amino acid longer than the hybrid enzyme. To facilitate cloning, the upstream region of *lacZ* has been further modified to contain a large multiple-cloning site derived from pUC18 (25) and pMTL20 (S. P. Chambers, D. A. Barstow, and N. P. Minton, Abstr. Int. Congr. Microbiol. 1986, P.13-12, p. 199). An artificial *trpA* terminator was synthesized and inserted upstream of the multiple-cloning site but was insufficient to prevent readthrough from promoters located in the pBR322 region of the plasmid. A second synthetic *trpA* terminator [Pharmacia (Canada) Inc., Baie d'Urfe, Quebec, Canada] was inserted in tandem with the other terminator at the unique *Nru*I site. The latter was created as a result of the addition of the first terminator. This arrangement proved successful in eliminating readthrough transcription. The sequence from the beginning of the *lacZ* gene upstream through the synthetic terminators was confirmed by double-stranded dideoxy sequencing with the universal primer (24) and Sequenase (United States Biochemical Corp., Cleveland, Ohio) (Fig. 1).

pQF60 was constructed by using the promoterless alkaline phosphatase (*phoA*) gene from pCB267 (7, 21). The alkaline phosphatase gene was transferred first to pBR322 and then to pQF40, replacing the promoterless *tetA* gene.

pQF70 was similarly created by using the luciferase (*luxAB*) genes taken from pLAV1, which was a gift from T. Baldwin (personal communication). The *luxAB* genes were first transferred to pBR322 and then to pQF40, replacing the promoterless tetracycline gene (Fig. 2).

Testing of promoter-probe vectors. All three vectors were linearized within the multiple-cloning region at a unique *Bam*HI restriction site, using enzyme and buffers purchased from Gibco/BRL Canada (Burlington, Ontario, Canada). An electroelution-purified 665-base-pair *Sau*3A restriction digest fragment from pBR322, containing the divergently arranged *tetA*/*tetR* promoters (2), was ligated into the linearized vectors. Insertion of the *Sau*3A fragment in either orientation should therefore activate the promoterless genes. Ligated plasmids were transformed into *E. coli* JM106 (25) by the method described by Maniatis et al. (15) and into *P. aeruginosa* OT684 (19) by the method described by Berry and Kropinski (4). All clones were selected on plates containing tryptic soy broth (Difco Laboratories, Detroit, Mich.)-supplemented agar (15 g/liter) and with either 150 μg of ampicillin or 300 μg of carbenicillin per ml for selection in *E. coli* and *P. aeruginosa*, respectively. The chromogenic substrates 5-bromo-4-chloro-3-indolyl-β-D-galactopyrano-

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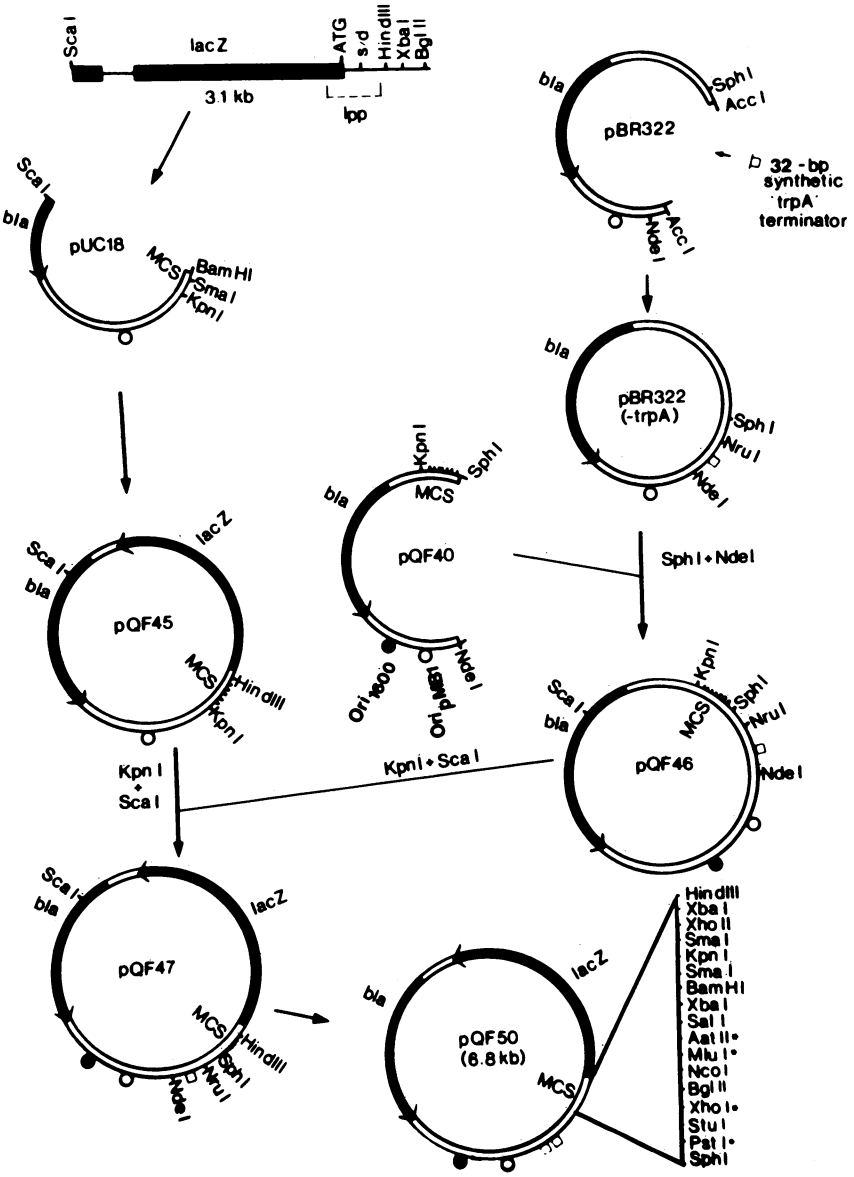

FIG. 1. The *lacZ* gene from pCB267 was excised as a 3.1-kilobase *Bgl*II-*Sca*I fragment and ligated into pUC18 cut with *Bam*HI-*Sca*I, creating pQF45. The *Bam*HI-*Bgl*II fusion creates a new *Xho*II site. A 32-base-pair *trpA* transcription terminator,

5′-CGCGAAAAAAAGCCCGCTCATTAGGCGGGCT
GCTTTTTTTCGGGCGAGTAATCCGCCCGATA-5′

was synthesized and inserted into pBR322 cut with *Acc*I. This insertion created a new *Nru*I site. The terminator was then transferred to pQF40 as a *Sph*I-*Nde*I fragment, creating pQF46. The *lacZ* gene from pQF45 was then transferred to pQF46 as a *Kpn*I-*Sca*I fragment, creating pQF47. On testing pQF47 in *E. coli*, a low level of β-galactosidase activity was detectable. To alleviate this problem a second synthetic *trpA* terminator was installed in tandem next to the first by blunt-end ligation into the *Nru*I site. This created the 6.8-kilobase vector pQF50. *, Nonunique restriction sites in the multiple-cloning site.

side (X-Gal) (Gibco/BRL) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co., St. Louis, Mo.) were added to the media at 20 and 40 μg/ml, respectively. Promoter-positive clones of pQF50 (*lacZ*) and pQF60 (*phoA*) manifested themselves as blue colonies. Luciferase-positive clones of pQF70 (*luxAB*) were detected by swabbing the inside of the petri dish lid with *n*-decanal (Sigma). This volatile long-chain aldehyde is utilized along with oxygen and FMNH by bacterial luciferase to produce a blue-green light that can be detected in a darkened room or by brief exposure to X-ray film (22). Positive and negative clones from all three constructs were screened for the 665-base-pair insert by mini-preps (5) and by digestion with *Sau*3A. Positive clones showed the insert, while negative clones revealed only religated vector without any insert. The orientations of the inserts were also determined by restriction endonuclease digestion. Clones demonstrating both promoter orientations were chosen from each plasmid type for *E. coli* and *P. aeruginosa* and subsequently assayed quantitatively for the activity of the reporter gene product. Assays for β-galactosidase were carried out with *o*-nitrophenol-β-D-galactopyranoside substrate (Sigma) as described by

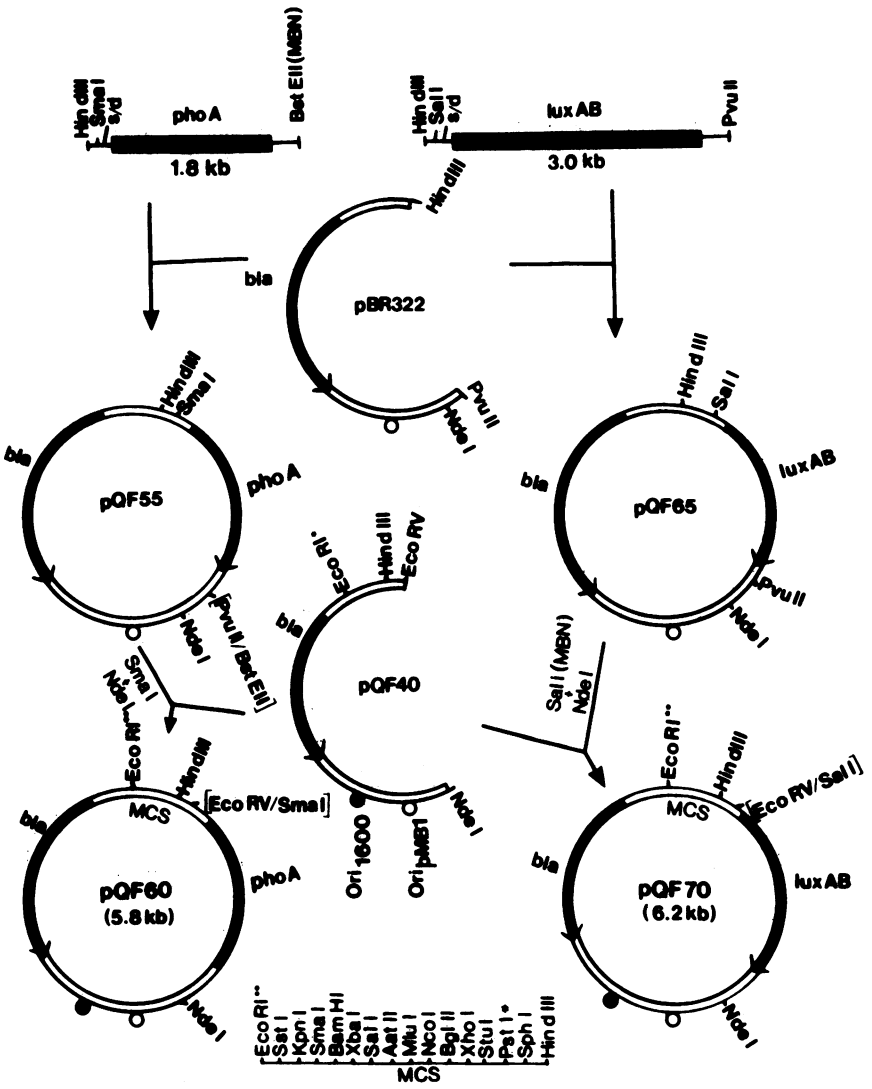

FIG. 2. The 1.8-kilobase alkaline phosphatase gene from pCB267 was recovered by digestion with *Bst*EII (rendered blunt with mung bean nuclease [MBN]) and *Hin*dIII. This was then ligated to pBR322 cut with *Hin*dIII-*Pvu*II, creating pQF55. The [*Pvu*II/*Bst*EII] fusion destroyed both restriction sites. The *phoA* gene was then transferred as a *Sma*I-*Nde*I fragment to pQF40 cut with *Eco*RV-*Nde*I, creating the final product, pQF60, which is 5.8 kilobases and contains a large multiple-cloning site (MCS). *Eco*RI** indicates the presence of a T1 transcription terminator (6) which prevents readthrough from upstream promoters into the *phoA* gene. The 3.0-kilobase luciferase gene (*luxAB*) was excised from pLAV1 as a *Hin*dIII-*Pvu*I fragment and ligated into pBR322 cut with *Hin*dIII-*Pvu*II, creating pQF65. The *luxAB* genes were then transferred from pQF65 by digestion with *Sal*I (rendered blunt with mung bean nuclease) and *Nde*I to pQF40 cut with *Eco*RV-*Nde*I. This created the final product, pQF70, which is 6.2 kilobases and has the same multiple-cloning site as pQF60. *, Nonunique restriction sites in the multiple-cloning site.

Miller (16), except that cells were broken by sonication (60 s on ice) instead of by permeabilization with toluene. Alkaline phosphatase activity was measured by using *p*-nitrophenol phosphate substrate (Sigma) as described by Kreuzer et al. (13) by using whole cells grown in phosphate-rich medium (LB plus 2 mM K_2HPO_4) to repress chromosomal alkaline phosphatase production. Luciferase was assayed using sonicated cell extracts and the buffer systems described by Baldwin et al. (3). Luminescence was quantitated with a scintillation counter (RackBeta; LKB Instruments, Inc., Rockville, Md.) set in the chemiluminescence detection mode. The activity of the culture was reported in the units described by Engebrecht et al. (8).

The data presented in Table 1 are comparable to results previously reported for the *tetA/tetR* promoter element from pBR322 (7, 21). The approximately twofold-greater strength of *tetR* over *tetA* is apparent in both *E. coli* and *P. aeruginosa* when all three reporter gene systems are used. Additionally, the values obtained for *E. coli* and *P. aeruginosa* are comparable when the copy number difference is taken into account. The copy number remained unchanged from the previously reported values of approximately 13 in *P. aeruginosa* and 36 in *E. coli* (9).

The vectors described are suitable for the detection and analysis of promoters in a wide range of gram-negative bacteria. Comparisons of promoter strength in different hosts are also possible. All the vectors have translational stop codons in all three reading frames located between the multiple-cloning site and the translation initiation codon of the indicator gene. This prevents initiation of transcription

TABLE 1. Expression of reporter genes *phoA*, *lacZ*, and *luxAB* in promoter selection vectors under the control of the *tetA* and *tetR* promoters

Host strain and relevant genotype	Promoter[a]	Sp act		
		β-Galactosidase[b] (pQF50)	Alkaline phosphatase[c] (pQF60)	Luciferase[d] (pQF70)
E. coli JM106 (*phoA*$^+$ *lacZ*)	None	<1	5	8
	tetA	85	98	446
	tetR	195	202	1,371
P. aeruginosa OT684 (*phoA*$^+$ *lacZ*)	None	1	1	2
	tetA	35	38	215
	tetR	60	79	640

[a] Promoter with which gene was aligned.
[b] Expressed in Miller units (16).
[c] Expressed in units as defined by Kreuzer et al. (13).
[d] Expressed in relative light units as described by Engebrecht et al. (8).

from other start sites into the indicator gene, which may have detrimental effects on its expression. These plasmids, like their predecessors pQF26 and pQF40 (9), are extremely stable even in the absence of antibiotic selection pressure.

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